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Optimization of Marine Bacteria *Enterococcus* sp. Biomass Growth by using Response Surface Methodology

S. Rajeshkumar[1,2], G. Gnana Jobitha[1,2], C. Malarkodi[1], C. Kannan[2,*], G. Annadurai[1,*]

[1] *Environmental Nanotechnology Division, Sri Paramakalyani Centre for Environmental Sciences, Manonmaniam Sundaranar University, Tirunelveli, TN, India.*
[2]*Department of Chemistry, Manonmaniam Sundaranar University, Tirunelveli, TN, India.*

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Abstract

Enterococcus sp. is one of the most important causes of substantial infections worldwide. It is a fastidious micro organism with fine nutritional and environmental requirements to cultivate, a characteristic that prevents the development of useful animal models to study the biology of the micro organism. This study was designed to determine optimal conditions for culture and growth of *Enterococcus* sp. The bacteria *Enterococcus* sp. was selected from isolates of marine water. Response surface methodology was employed to optimize a bacterial biomass growth. The five variables involved in the study of growth conditions were Yeast extract, beef extract, NaCl concentration, pH and Temperature. This is an estimate of the fraction of overall variation in the data accounted by the model, and thus the model is capable of explaining 99.96% of the variation in response

Keywords : *Enterococcus sp.; Growth, Optimization ; Response Surface Methodology.*

1. INTRODUCTION

Response surface methodology (RSM) is a compilation of mathematical and statistical techniques useful for emergent, improving, and optimizing processes. It is a well-known method applied in the optimization of composition in the medium and other critical variables responsible for the fabrication of biological molecules (Myers and Montgomery 2007). For the rapid optimization of microbiological media RSM is vigorously involved (Maddon and Richard 1977). Marjory it plays an important role in the production of enzymes using microorganisms like cyclodextrin lucanotransferase from *Bacillus stearothermophilus* HR1 (Rahman et al. 2004), lucosyl transferase by *Aspergillus niger* Lee and Chen 1997). linolenic acid in *Mortierella ramanniana* var. *ramanniana (*Dyal et al. 2005) alpha amylase production by *Bacillus amyloliquefaciens (*Dhanya et al. 2008*)*, Nattokinase production by *Bacillus subtilis (*Deepak et al., 2008*)* chitinase production by *Pantoea dispersa* (Gohel et al. 2005) cyclodextrin glycosyltransferase production from *Klebsiella pneumoniae* AS-22. (Gawande and Patkar 1999), alkaline protease from *Bacillus horikoshii* (Joo et al. 2002), thermostable alkaline protease from thermophilic and alkalophilic *Bacillus* sp. JB-99 (Johnvesly and Naik 2001) were produced previously. There are many designs playing an important role in the increasing of biomass growth. In that Fractional factorial design provides an option when the numbers of runs for a full factorial design is too large to be feasible.

Plackett–Burman design, Taguchi design, central composite design and Box–Behnken design are fractional factorial designs that were used for increased microbial production processes. In that Box– Behnken design provides an inexpensive option to the central composite design(Jianlong and Wei 2008). Optimizing of biomass growth is one of the important processes in microbiology

*C.Kannan. Tel.: +919443507036
E-mail : chellapandiankannan@gmail.com

and microbial technology having a wide variety application. There are many kind of secondary metabolites are isolated from marine bacterial strains (Jensen and Fenical 1994). In this investigation we produced the maximum growth of *Enterococcus* sp by using Response surface methodology.

2. MATERIALS AND METHODS

2.1 Media, chemical and Microorganism

Microbiological nutrients Yeast extract, Beef extract and sodium chloride were purchased from Hi Media, Mumbai. The marine water sample was collected from Kanyakumari coastal area, Kanyakumari district, South Tamilnadu, India in a clean sample container. A bacterial strain was isolated by serial dilution technique and identified by biochemical tests.

2.2 Selection of significant variables by Box-Behnken design

Response surface methodology (RSM) consists of a group of empirical techniques devoted to the evaluation of relations existing between a cluster of controlled experimental factors and the measured responses, according to one or more selected criteria. For the selection of significant variables for optimization of Maximum Biomass Activity by bacterial strain *Enterococcus sp*, a variety of Yeast Extract (g/L), Peptone (g/L), NaCl (g/L), Temperature (^{o}C) and pH were tested and identified via the Box-Behnken design experiments. A total of five parameters were included for selection, with each variables represented at three levels (-1, 0, +1). The variables were as follows: peptone (g/dL) (0.1, 0.5 0.9) (X_1); Yeast extract (0.15, 0.3, 0.45) (g/dL) (X_2), NaCl (g/dL) (0.1, 0.5, 0.9) (X_3), pH (6.3, 7.05, 7.8.) (X_4) and Temperature (25, 30, 35 ^{0}C) (X_5) at different concentrations of above nutrient designed (Design expert 7.0.1.) as shown. The design experiments were carried out in conical flasks containing minimal medium and inoculated with bacterial strain *Enterococcus* sp at 180 rpm in shaker for different incubation period (hrs). After incubation period (hrs), the amount of maximum Biomass Activity was calculated by taking samples. The principal effects of each variable on maximum biomass activity were estimated as the difference between both averages of measurements made at the higher level and at the lower level. The significance of each variable was determined via Student's t-test. The statistical software package 'Design Expert@ 7.0.1. Stat Ease Inc., (Minneapolis, MN. USA) was used to analyze the experimental design. At certain time intervals, 5 ml of sample was withdrawn, analyzed with a UV–Vis spectrophotometer and returned to the reactor. The Maximum Biomass Activity was measured with the above-mentioned spectrophotometer at 600 nm. Calibration plot based on Beer–Lambert's law was established by relating absorbance to the concentration.

2.3 Experimental design and optimized by response surface methodology

RSM is an empirical statistical modeling technique employed for multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariable equations simultaneously. RSM is used to determine the optimum response of cells, for the biosynthesis of nanoparticles under a wide range of nutrient conditions. Basically this optimization process involves three major steps, performing the statistically designed experiments, estimating the coefficients in a mathematical model, predicting the response and checking the adequacy of the model (Box and Hunter 1957; Box and Behnken 1960).

In order to describe the effect of factors on response surface in the region of investigation, a Box-Behnken design with five levels was performed. A Box - Behnken design in the five independent variables were used to obtain the combination of values that optimize the response within the region of three dimensional observation spaces, which allows one to design a minimal number of experiments (Cochran and Cox 1968; Gopinath et al. 2003). The Box-Behnken design was applied using Design-Expert (version 7.0.1) and the total number of experiments with four variables was 46 experiments. Fourty experiments were augmented with ten replications at the center points to evaluate the pure error. In the optimization process the response can be related to chosen factors by linear or quadratic models. The average of the maximum biomass activity of the duplicate

values obtained was taken as dependent variable or response Y_i (U). Duplicates are necessary to estimate the variability,of experimental measurements, i.e. the repeatability of the phenomenon. Replicates at the centre of the domain in three blocks permit the checking of the absence of bias between several sets of experiments. The experimental results of RSM were fitted via the response surface regression procedure, using the following second–order polynomial equation:

$$Y = f(X_1, X_2, X_3, X_4, X_5 \ldots\ldots X_k) \quad (1)$$

The true relationship between Y and X_k may be complicated and in most cases, it is unknown; however, a second-degree quadratic polynomial can be used to represent the function in the range of interest:

$$Y = R_o + \sum_{i=1}^{k} R_i X_i + \sum_{i=1}^{k} R_{ii} X_i^2 + \sum_{i=1,i<j}^{k-1} \sum_{j=2}^{k} R_{ij} X_i X_j + \varepsilon \quad (2)$$

where X_1, X_2, X_3, X_4, X_5, …, X_k are the input variables which affect the response Y, R_0, R_i, R_{ii} and R_{ij} (i = 1–k, j = 1–k) are the known parameters, ε is the random error. A second-order model is designed such that variance of Y is constant for all points equidistant from the center of the design. Coding of the variables was done according to the following equation

$$X_i = \left(\frac{X_i - X_o}{\Delta X_i} \right) \quad (3)$$

Where X_i is the coded value and X_o is the actual value of the independent variable, X_o is the actual value at the center point and ΔX_i is the step change value. The following equation was used for coding the actual experimental values of the factors in the range of (1- to +1). In system involving five significant independent variables X_1, X_2, X_3, X_4 and X_5 the mathematical relationship of the response of these variables can be approximated by quadratic (second degree) polynomial equation;

$$Y = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_4 X_4 + b_5 X_5 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^3 + b_{44} X_4^2 + b_{55} X_5^2 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{14} X_1 X_4 + b_{15} X_1 X_5 + b_{23} X_2 X_3 + b_{24} X_2 X_4 + b_{25} X_2 X_5 + b_{34} X_3 X_4 + b_{35} X_3 X_5 + b_{45} X_4 X_5$$

Where Y is the predicted value, b_o is the constant, Yeast Extract (g/L) (0.5, 1.5, 2.5) (X_1); Peptone (g/L) (0.5, 1.5 2.5) (X_2), NaCl (g/L) (0.5, 1.5, 2.5) (X_3), Temperature (35, 30, 40^{0}C) (X_4) and pH (6.0, 7.4, 8.8.) (X_5), b_1, b_2, b_3, b_4 and b_5 are linear coefficients, b_{12}, b_{13}, b_{14}, b_{15}, b_{23}, b_{24} b_{25}, b_{34}, b_{35} and b_{45} are cross product coefficients and b_{11}, b_{22}, b_{33} b_{44} and b_{55} are quadratic coefficients. The design of experiments was carried out for analysis using the design expert by Stat Ease lnc, Statistics Made Easy, Minneapolis, MN Version .7)

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of microorganism:

The microorganism in the marine water sample was isolated by serial dilution technique. The dominant bacterial strains are identified as *Enterococcus* sp. on the morphological and biochemical the characteristic describing the organism found to be *Enterococcus* sp. wasdetermined using Bergey's manual of determinative bacteriology (Holt et al. 1994)

3.2 Optimization by response surface methodology

The experimental conducted in the present study were targeted toward the construction of a quadratic model consisting of forty six trials. The design matrix and the corresponding results of RSM experiments to determine the effects of five independent variables peptone (g/L) (0.1, 0.5, 0.9) (X_1); yeast extract (g/L) (0.15, 0.3, 0.45) (X_2), NaCl (g/L) (0.1, 0.5, 0.9) (X_3), pH (6.3, 7.05, 7.8) (X_4) and Temperature (25, 30, 35^{0}C) (X_5), are shown, along with the mean predicted values. The fisher F-test with a very low probability value (P model > F= 0.0001) demonstrate a very high significance for the regression model.

The goodness of fit of the model was checked by the determination coefficient (R^2). The ANOVA analysis of the optimization study indicated that the model terms, linear $PX_1 < 0.0001$, $PX_2 < 0.0001$, $PX_3 < 0.0001$, $PX_4 < 0.0001$, PX_5 0.0074; quadratic $PX_{11} < 0.0001$, $PX_{22} < 0.0001$, PX_{33} 0.0051, $PX_{44} < 0.0001$, $PX_{55} < 0.0001$ and 6 interaction PX_1X_2 0.058,, $PX_1X_5 < 0.0001$, PX_2X_5 0.008, $PX_3X_4 < 0.0001$, PX_3X_5 0.3852, PX_4X_5 0.002 c

Table 1. Regression analysis for the maximum Biomass Activity for quadratic response surface model fitting (ANOVA)

Source	Co-efficient estimate	df	Mean Square	F Value	p-value Prob > F
Model	0.633833	20	0.195543292	977.7132	< 0.0001
X_1-Peptone	0.090125	1	0.12996025	649.7991	< 0.0001
X_2-Yeast Extract	0.06075	1	0.05904898	295.2439	< 0.0001
X_3-NaCl	0.078937	1	0.099698036	498.4885	< 0.0001
X_4-pH	0.08225	1	0.108241	541.2032	< 0.0001
X_5Temperature	0.010313	1	0.001701563	8.507784	0.0074
X_1X_2	0.014	1	0.000784	3.919987	0.0588
X_1X_3	-0.05875	1	0.01380625	69.03102	< 0.0001
X_1X_4	-0.03	1	0.0036	17.99994	0.0003
X_1X_5	-0.06125	1	0.01500625	75.031	< 0.0001
X_2X_3	-0.07	1	0.019599977	97.99956	< 0.0001
X_2X_4	0.1295	1	0.067081	335.4039	< 0.0001
X_2X_5	0.027	1	0.002916	14.57995	0.0008
X_3X_4	-0.21025	1	0.17682025	884.0983	< 0.0001
X_3X_5	-0.00625	1	0.00015625	0.781247	0.3852
X_4X_5	-0.03025	1	0.00366025	18.30119	0.0002
X_1^2	0.098937	1	0.085428028	427.1387	< 0.0001
X_2^2	0.129938	1	0.147349149	736.7433	< 0.0001
X_3^3	0.014688	1	0.001882673	9.413334	0.0051
X_4^4	0.550937	1	2.649007637	13244.99	< 0.0001
X_5^5	-0.07165	1	0.04479819	223.9902	< 0.0001
Residual	0.005000017	25	0.000200001		
Lack of Fit	0.005000017	20	0.000250001		
Pure Error	0	5	0		
Cor Total	3.915865864	45			

Data analysis using the statgraphics software at 95% of confidence level permitted to obtain a semi-empirical expression which consists of 15 statistically significant coefficients having absolute value greater than zero, with a probability of 95% ($p < 0.05$): By means of Multi Linear Regression method (Box and Hunter 1957; Box and Behnken 1960) a quadratic regression equation was developed based on statistical experimental design. (Cochran and Cox 1968). The significance of all regression co-efficient was checked by means of Student's t-test. The final regression model in terms of coded factors is presented as follows:

$$Y = 0.633 + 0.090\,X_1 + 0.06X_2 + 0.078\,X_3 + 0.022\,X_4 + 0.010\,X_5 + 0.098\,X_1^2 + 0.129\,X_2^2 + 0.041\,X_3^3 + 0.550\,X_4^2 - 0.076\,X_5^2 + 0.014\,X_1X_2 - 0.058\,X_1X_3 - 0.03\,X_1X_4 - 0.061\,X_1X_5 - 0.07\,X_2X_3 + 0.129\,X_2X_4 + 0.027\,X_2X_5 - 0.021\,X_3X_4 - 0.006\,X_3X_5 - 0.030\,X_4X_5 \quad (5)$$

The goodness-of-fit of the regression model can be ascertained by applying the Fischer F-test. This model explains perfectly the experimental range studied(R^2 adjusted = 0.9977). The results of analysis of variance (ANOVA) are shown which indicate thatthe were significant ($P < 0.05$). Other interaction terms

were negleted.predictability of the model is at 95% and 99% confidence interval. The regression equation obtained from the ANOVA showed that the R^2 (multiple correlation co-efficient) was 0. 9987 (a value > 0.61% indicated fitness of the model). This is an estimate of the fraction of overall variation in the data accounted by the model, and thus the model is capable of explaining 99.96% of the variation in response. The adjusted R^2 is 0. 9977 and the predicated R^2 is 0. 9987, which indicates that the model is good (the range of the R^2 values 0-1.0 and nearer to 1.0 the value is the more fit the model). The adequate precision value of the present model was 71.51.

3.3 Response surface (contour) plots and optimization conditions

After performing a screening of factors and their interactions, the response surface analysis was carried out, in order to find the optimal conditions for the maximum biomass activity. Response surface plots provide a method to predict the nitrogen, salt, pH and Temperature variables necessary to achieve a complete disappearance maximum biomass activity for different values of the test variables. In addition, the contours of the plots help in identification of the type of interactions between the selected variables. Each contour curve represents an infinite number of combinations of the two selected variables with the other maintained at their respective zero coded level. A circular contour of response surfaces indicates that the interaction between the corresponding variables is negligible. An elliptical or saddle nature of the contour plots indicates that the interaction between the corresponding variables is significant.

3.4 Effect of peptone

The contour plot represents maximum biomass activity against peptone and yeast extract. The biomass activity (0.860 (OD-ABS) at a particular range of peptone (0.10 to 0.90 g/L) and yeast extract (0.1 to 0.45 g/L) which is also clearly illustrated in Fig.1. The optimum level of biomass activity occurs with 98% at Peptone (0.45 g/L) and yeast extract (0.30 g/L) calculated by derivatization of the equation (3) and by solving the inverse matrix. Peptone as the major source playing a mojor role in the improving the antibiotic activity of *Xenorhabdus nematophila* TB was proved (Fang et al. 2010).

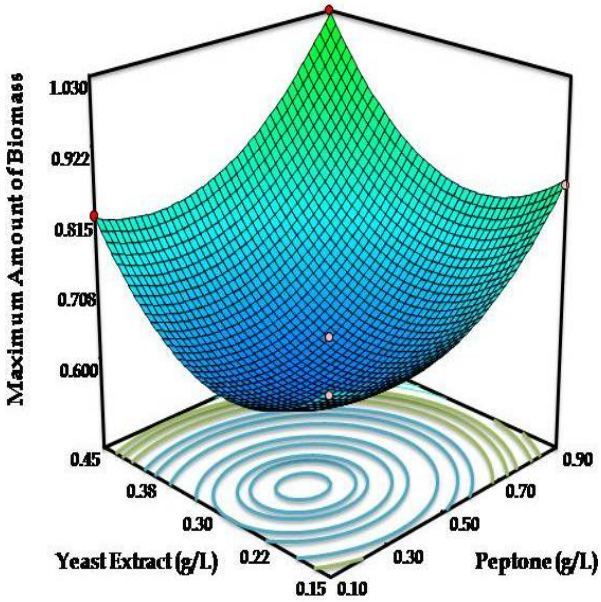

Fig. 1 : Maximum biomass activity on 3 D graphics for response surface optimization versus Peptone and Yeast Extract.

3.5 Effect of yeast extract

From Fig.2 the maximum biomass activity was found to occur with Yeast extract (0.15 to 0.45 g/L) and sodium chloride (0.1 to 0.9 g/L) at the level of biomass activity (98%). Optimum level of yeast extract 0.45 g/L and sodium chloride 0.9 g/L showed the maximum biomass activity as (0.880 (OD-ABS). The concentration of yeast extract in minimal medium (Fig.2) was varied from (0.15 to 0.45 g/L) and there was no considerable increase in the biomass activity beyond 0.15 g/L. Increasing the concentration of yeast extract from 0.05% to 0.15 g/L increased the biomass activity from 0.530 to 0.880 (OD-ABS). But above 0.3 g/L yeast extract, the biomass activity reaches a plateau and there is no considerable increase in the biomass activity beyond 1.5%. Increasing the initial yeast extract concentration not only increases the bacterial yield, but also the time required for completion of the biomass activity. A number of substances have been reported in the literature that

enhances the growth ofenhances the growth of *Geotrichum candidum* and likely constituents of nitrogen sources (Gopinath et al. 2003).

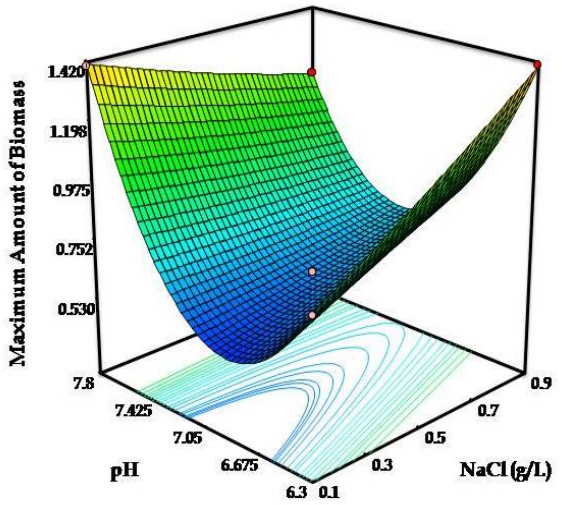

Fig. 3 : Maximum biomass activity on 3 D graphics for response surface optimization versus NaCl and pH

3.6 Effect of sodium chloride

Figure. 3, clearly indicate the maximum biomass activity (1.420 (OD-ABS) with NaCl (0.1 to 0.9 g/L) and pH (6.3 to 7.8) were clearly seen. Bacterial cells maintain an internal osmotic pressure at about 0.45 g/L solution of NaCl. If the environment has a lower osmotic pressure than the cell (hypotonic), water tends to penetrate into the cell, and higher extra cellular osmotic pressure (hypertonic) causes the protoplasm to lose water through the partially permeable cell membrane (Annadurai et al. 2002). Salt was playing a major role in the growth of bacterial culture and the salinity determines the growth ranges. (Bhat et al. 1983; Liew et al. 2005).

3.7 Effect of pH

3 D plot representing maximum biomass activity (1.270 (OD-ABS) against pH (6.2to 7.8) and Temperature (25 to 35°C) is shown in Fig.4. Optimization level of pH (7.05) and Temperature (25°C) were determined at maximum biomass activity. The lactic acid production values increased of 170% when the pH of the supplement hydrolysate was controlled (Lima *et al*., 2010*)*. The keratinase was active in alkaline condition, with optimal activity at pH 8.0 and the activity was declined as the pH augmented above the optimum. (Anbu et al. 2005).

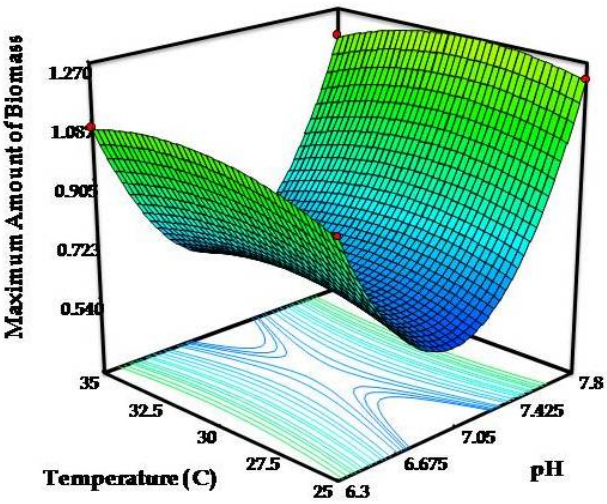

Fig. 4 : Maximum biomass activity on 3 D graphics for response surface optimization versus pH and Temperature

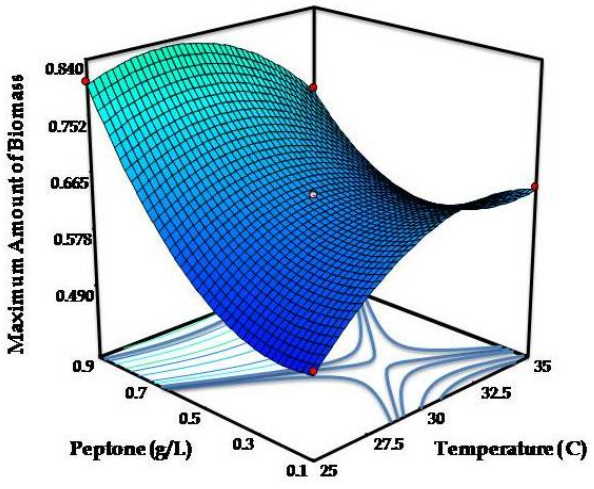

Fig. 5 : Maximum biomass activity on 3 D graphics for response surface optimization versus Temperature and Peptone.

3.8 Effect of temperature

3 D plot representing maximum biomass activity (0.840 (OD-ABS) temperature (25 to 35^{0}C) and peptone (0.1 to 0.9 g/L) is shown in Fig.5. Optimization level of temperature (25^{0}C) and peptone (0.45 g/L) were determined maximum production of bacterial growth. Temperature exerts a significant regulatory influence on the rate of metabolism. The bacterial activity rapidly reduces at temperatures below the finest temperature range, whereas the bacterial activity is not being affected much by temperature change within the optimum temperature range was proved in enzyme and lactic acid production(Annapurna et al. 2009**,** Lima et al. 2010).

4. CONCLUSION

The use of Response Surface Methodology as a statistical tool to develop the growth of *Enterococcus* sp. in nutrient medium has been demonstrated in this study In this all the parameters like nitrogen sources, salt concentration, pH and temperature were vigorously involved and the maximum production of culture level was established. In future this result will be very useful for the maximum production of enzymes, antibiotics, secondary metabolites and nanoparticles synthesis. The adjusted R^2 is 0. 9977 and the predicated R^2 is 0. 9987, which point out that the representation is good. The adequate exactitude value of the present model i-s 71.51.

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